Severe Case of Rickettsiosis Identified by Metagenomic Sequencing, China

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A case of *Rickettsia sibirica* subspecies *sibirica* BJ-90 infection in China was identified by metagenomic analysis of an eschar biopsy specimen and confirmed by nested PCR. Seroprevalence of spotted fever group *Rickettsia* was ≈17.4% among the local population. This report highlights the threat of rickettsioses to public health in the Qinghai–Tibet Plateau.

Rickettsia, mainly transmitted by ticks, are a group of obligate gram-negative bacteria that cause mild to life-threatening rickettsioses. Two main groups of *Rickettsia* have been described on the basis of genetic differences and pathology, spotted fever group (SFG) and typhus group (TG). In China, 5 members of SFG have been identified in human cases (*1*–*4*), and 7 kinds of *Rickettsia* have been detected from ticks or animals in the Qinghai–Tibet Plateau, including *R. heilongjiangensis, R. raoultii, R. slovaca,* and *R. sibirica*, which are known to be pathogenic to humans (*5*–*7*). However, clinical cases have not been reported. Thus, rickettsioses are probably neglected by local physicians and public health officers. We report a severe case of *R. sibirica* subspecies *sibirica* BJ-90 infection in this region.

A 50-year-old herdsman from Zhamashi, Qinghai Province, China, was hospitalized on July 13, 2018, because of intensive intermittent headache, anorexia, and chest tightness. On his fifth day of sheep shearing (designated as day 1), a blood-fed tick had been found on his head. The tick was removed by hand but its mouth parts remained in the man's scalp. The next day, he became ill with fever, myalgia, itchiness, and asthenia. On day 5, his symptoms intensified and included severe intermittent headaches, which lasted for ≈10 minutes at each onset; high fever, up to 39.5°C; and fatigue, palpitation, nausea, and vomiting. Erythematous rashes appeared on his trunk, all 4 limbs, and the area behind the ears. Because signs of neurologic dysfunction, including confusion, drowsiness, and delirium appeared, he sought care at Qilian County Hospital on day 9, where he was treated for infectious endocarditis for 3 days before transfer to Qinghai State Hospital. During his visit at the Qinghai State Hospital, he was conscious and alert. Erythematous macules were observed over his trunk, elbow, and lower limbs. A 1.5 × 1.1 cm^2 black eschar was visible at his right posterior occipital bone area; no tenderness was reported (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/27/5/20-3265-App1.pdf). The eschar was surgically excised on day 16. No lymphadenopathy was found.

Alterations of the patient's blood biochemistry included increased neutrophils (88.5% [reference 45%–75%]); decreased lymphocytes (9.3% [reference 20%–50%]), eosinophils (0% [reference 0.4%–8%]), and monocytes (1.9% [reference 3%–10%]); elevated creatine kinase–MB (42 U/L [reference 0–25 U/L]) and lactate dehydrogenase (445 U/L [reference 110–245 U/L]); and highly increased C-reactive protein (97.1 mg/dL [reference 0–5 mg/dL]), procalcitonin (0.433 ng/dL [reference 0–0.046 ng/dL]), D-dimers (12.28 μg/mL [reference 0–1.5 μg/mL]), fibrinogen degradation products (25 μg/mL [reference 0–5 μg/mL]), and β-microglobulin (4.5 μg/mL [reference 0.8–1.8 μg/mL]). The patient was prescribed levofloxacin lactate (0.5 g/d for 6 d). His symptoms subsided, and he was discharged on day 20.

On the basis of tick-bite history and the triad clinical characteristics (fever, rash, and eschar), nested PCR targeting the rickettsial citrate synthase conserved gene (*glt*A) was performed by using the eschar DNA as a template (Appendix). The 547-bp amplicon sequence shared 100% identity to *R. sibirica* 246, *R. sibirica* subsp. *sibirica* BJ-90, and *R. sibirica* subsp. *mongolitimonae* HA-91. The eschar DNA was sequenced by next-generation sequencing (BGI Genomics, https://www.bgi.com). A total of 21.6 Gb clean data were recovered from the high-throughput sequencing. Human reads (accounting for 99.9%) were filtered out. The remaining reads were mapped on the genome of *R. sibirica* 246 (GenBank accession no. AABW0100000). Rickettsial unique reads (n = 266) were analyzed against Refseq (https://www.ncbi.nlm.nih.gov/refseq; taxid 766). Most (226/266 [85%])

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Table. Homology of 266 identified rickettsial unique reads shared by *Rickettsia* species, China*

% Identity	No. reads			
	BJ-90	*R. sibirica* 246†	HA-91‡	*R. heilongjiangensis*‡
100	226	213	153	48
99	25	35	76	65
98	6	9	19	46
1–97	9	9	17	102
0	0	0	1	5

*BJ-90, *R. sibirica* subsp. *sibirica* BJ-90; HA-91, *R. sibirica* subsp. *mongolitimonae* HA-91.
†No significant difference (p = 0.35) compared with BJ-90 by Wilcoxon ranked nonparametric test.
‡Significant difference (p<0.05) compared with BJ-90 by Wilcoxon ranked nonparametric test.

reads were 100% identical to *R. sibirica* subsp. *sibirica* BJ-90, whereas 213/266 (80%) were identical to *R. sibirica* 246, indicating that the *Rickettsia* was closest to *R. sibirica* subsp. *sibirica* BJ-90 (Table; Appendix Figure 2). Partial sequences of outer membrane protein A, outer membrane protein B, 17 kDa lipoprotein, surface cell antigen 1, and surface cell antigen 4 were amplified with specific primers. Phylogenetic trees showed that the Qinghai sequences clustered with *R. sibirica* subsp. *sibirica* BJ-90 (Figure; Appendix Figure 3). On the basis of next-generation sequencing data and PCR results, we concluded that the causative agent of the patient's infection is closely related to *R. sibirica* subsp. *sibirica* BJ-90.

We evaluated serum samples from the patient and persons from his surrounding community. Antibodies against *R. rickettsii* (SFG) and *R. typhi* (TG) were determined by indirect immunofluorescence assay. IgG titers of the patient's paired serum samples on day 13 (1:128) and day 167 (1:4,096)) against SFG were increased by ≥4-fold, suggesting a recent infection with SFG. Approximately 17.4% (4/23) of the serum

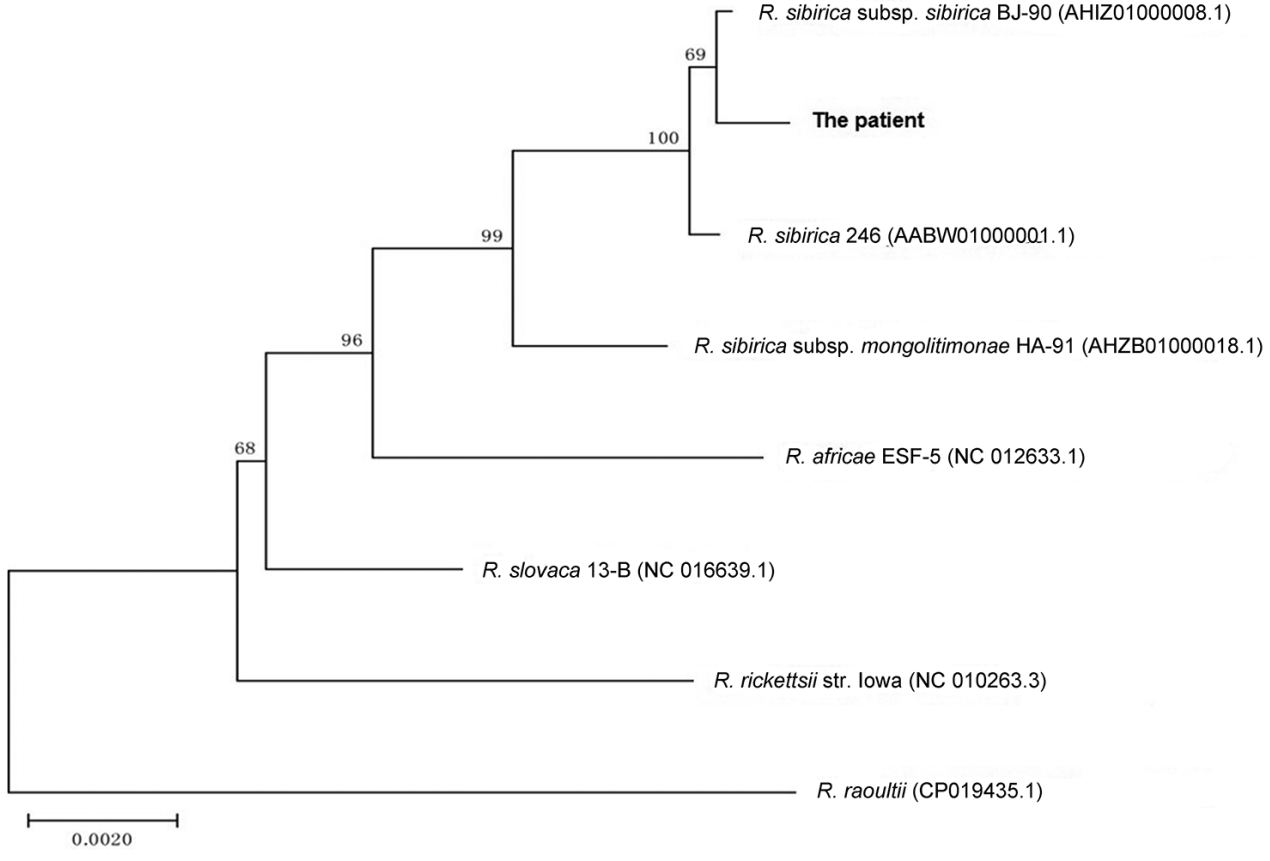

Figure. Phylogenetic analysis of concatenated nucleotide sequences from *Rickettsia* species collected in 2018 from eschar DNA from a patient in Qinghai Tibet Plateau, China (boldface), and reference sequences. A phylogenetic tree was constructed on the basis of the concatenated partial *glt*A, *omp*A, *omp*B, 17 kDa, *sca*1, and *sca*4 nucleotide sequences by using the neighbor-joining method with 1,000 bootstrap replicates. Numbers >70 indicate the bootstrapping value. GenBank accession numbers listed in Appendix Table 3 (https://wwwnc.cdc.gov/EID/article/27/5/20-3265-App1.pdf). Scale bar represents nucleotide substitutions.

samples from the local community were positive for SFG, and 4.3% (1/23) were positive for TG (Appendix Table 1), indicating a high seroprevalence of SFG and co-circulation of TG in the region.

Because of the treating physicians' unawareness of the prevalence of rickettsioses, the patient's illness was misdiagnosed and incorrectly treated. In light of the fatal cases of *R. sibirica* subsp. *sibirica* infection recently documented in Russia and China (*8*–*10*), our report highlights the risk for rickettsial diseases among the public in the Qinghai–Tibet Plateau region and the urgent need for a large-scale seroepidemologic survey.

Acknowledgments

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Eosinophilic Meningitis and Intraocular Infection Caused by *Dirofilaria* sp. Genotype Hongkong

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Eosinophilic meningitis caused by human diroflarial infection is rare. We report a case of eosinophilic meningitis and concomitant intraocular dirofilarial infection in India. Sequencing of the mitochondrial genome identified the worm as *Dirofilaria* sp. genotype Hongkong, a close relative of *D. repens* nematodes.

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Appendix

Materials and Methods

Ethics statements

Experimental protocols for collecting human clinical samples and isolating *Rickettsiae* from the samples were approved by the Ethical Review Committee of the National Institute for Communicable Disease Control and Prevention (ICDC), Chinese Center for Disease Control and Prevention (China CDC). Written consent regarding any potential identifiable images or data included in this article was obtained from all participants before the study.

DNA isolation and metagenomic sequencing

The eschar specimen (≈0.5 × 0.4 × 0.4 cm^3) was washed with PBS twice, shredded, and homogenized with TissueLyser II (QIAGEN, USA) in up to 1 ml of PBS. Homogenate DNA (200 µL) was extracted (QIAamp Tissue kit, QIAGEN, USA). DNA aliquot were stocked at −80°C and 4°C until use. Quality and quantity of the DNA were evaluated by Nanodrop spectrophotometer (Thermo Scientific, USA).

A library of eschar DNA (2 µg) was constructed (BGI Genomics, China) and sequenced using BGIseq2000. The dataset was cleaned by removing the adaptors and low quality reads. Reads mapped to the hg38 genome were filtered out. The remaining reads were mapped to the reference genome of *R. sibirica strain* 246 using bowtie2 with default parameters. Sequence of the individual rickettsial reads was analyzed by blastn against NCBI Refseq Genome Database of *Rickettesias* (taxid:766). A phylogenetic tree was constructed using iqtree software on the basis

of the sequence of the concatenated rickettsial unique reads and their homologous segments in seven complete genomes of *Rickettsia*.

Molecular diagnosis and amplicon sequencing

Primers targeting rickettsial species-specific genes of citrate synthase (*glt*A) and outer membrane protein A (*omp*A), outer membrane protein B (*omp*B), 17kDa lipoprotein (17kDa), surface cell antigen1 (*sca*1), and surface cell antigen 4 (*sac*4) were verified using chromosomal DNA of *R. rickettsii* (Appendix Table 1). To avoid cross-contamination, positive controls were skipped when field samples were screened. Eschar DNA (200 ng/reaction) was used as template for PCR under condition of 95°C for 5 minutes (one cycle), 95°C for 20 seconds, 51–53°C for 20 seconds, 68°C for 30 seconds (30 cycles), followed by 68°C for 5 minutes. When PCR band was faint on agarose gel (loading 5 μL of PCR product), a second PCR was performed as described above using the original PCR product (2 μl) as template. The high fidelity PCR products were Sanger sequenced and used for blastn search. Phylogenetic trees were generated by the neighbor-joining method implemented in MEGA 6.06 software with bootstrap replicates of 1000.

Serologic test

The first blood sample was collected when the patient was admitted at the Qinghai State Hospital on day 13. A follow-up sample was obtained about 5 months after his release from hospital (on day 167). Serum samples from the surrounding community were collected (male = 6, female = 17; ages 6–75 years old). All sera were examined for IgG and IgM against SFGR (*R. rickettsii)* and TG (*R. typhi)* by indirect immunofluorescence assay (IFA) following the manufacturer's instructions (Focus Diagnostic Inc. Cypress, USA). The sera were 2-fold serial diluted starting at 1:2 and initially tested at dilution of 1:32 antibody titers were further determined for the blood samples that demonstrated strong fluorescence at the primary screening. A titer was defined as reciprocal of the last dilution that specific fluorescence can be observed (comparing with positive and negative control). A titer of ≥64 was designated as a positive exposure history of SFGR. A recent infection was considered if the titers of a person's paired serum samples, collected in 2 weeks apart, increased by ≥4-fold (Appendix Table 2).

Statistical analyses

Identities (%) of the 266 rickettsial reads matched to *R. sibirica* subsp. *sibirica BJ-90, R. sibirica 246, R. sibirica subsp. mongolitimonae* HA-91, and *R. helongjiangensis* were analyzed using Wilcoxon ranked nonparametric test by SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). A paired Student's *t*-test was performed using Microsoft Excel for statistical difference determination (p-value ≤ 0.05).

Nucleotide sequence accession number

Nucleotide sequences obtained in this study were deposited in the GenBank and the National Genomic Data Center (NGDC) (Appendix Table 3).

Reference

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Appendix Table 1. Titer of IgG to *R. rickettsii* and *R. typhi* among the local population in Zhamashi, Qilian county, Qinghai province*

Sample no.	Gender	Age	*R. rickettsia*	*R. typhus*
1	M	68	<32	<32
2	M	51	<32	<32
3	F	42	<32	<32
4	F	48	<32	<32
5	F	44	= 128	<32
6	M	6	<32	<32
7	F	37	= 128	<32
8	F	57	<32	<32
9	F	75	<32	= 128
10	M	64	<32	<32
11	F	71	<32	<32
12	F	61	<32	<32
13	F	70	<32	<32
14	F	27	<32	<32
15	F	26	<32	<32
16	F	30	= 64	<32
17	M	40	= 64	<32
18	M	61	<32	<32
19	F	46	<32	<32
20	F	72	<32	<32
21	F	58	<32	<32
22	F	50	<32	<32
23	F	42	<32	<32
The patient†	M	50	= 128	<32
The patient‡	M	50	= 4096	<32

*Blood samples (n = 23) were collected in the patient's local area, including 6 males and 17 females, ages 6–72, and determined by indirect immunofluorensence assay. Positive was defined when titer of IgG was ≥1:64.

†The patient's serum on day 13.

‡The patient's serum on day 167.

Appendix Table 2. Primers for PCR and sequencing

Primer	Target gene	Sequence (5′-3′)	Reference
R001F	*glt*A	ttgtcagtctactatcaccTATATA	This study
R001R		gaagcattctgctcatgatcggcat	This study
R002F	*omp*A	GGAGTAATGTAGCAGGTACCGCTAG	This study
R002R		ccgcccaatattaccggtttgag	This study
R005F	*omp*B	GTAACCGGAAGTAATCGTTTCGTAAA	This study
R005R		CTTTATAACCAGCTAAACCACCTT	This study
R008F	*sca*1	GAGAGTCTCATATAAAAAGAGG	This study
R008R		ATGTTCTACCGCTCCTTGG	This study
R010F	17kDa	GCTCTTGCAACTTCTATGTTACA	This study
R010R		CATTGTTCGTCAGGTTGGCGgCATG	This study
R011F	*sca*4	atgagtaaagacggtaacct	(*1*)
R011R		aagctattgcgtcatctccg	(*1*)

Appendix Table 3. Information of nucleotide sequences obtained in this study and GenBank accession numbers*

		Identity to (%)			
Targeting gene†	Amplicon size (bp)	BJ-90	246	HA-91	GenBank accession no.
*glt*A	547	100	100	100	MT521745
*omp*A	619	99.51	99.51	98.68	MT521746
*omp*B	512	99.80	99.41	99.02	MT521747
*sca*1	648	99.84	99.84	99.22	MT521748
17kDa	441	99.77	99.77	99.54	MT521749
*sca*4	931	99.24	99.13	98.36	MT521750
Reads‡	21600§	99.7	99.63	98.9	CRA002872¶

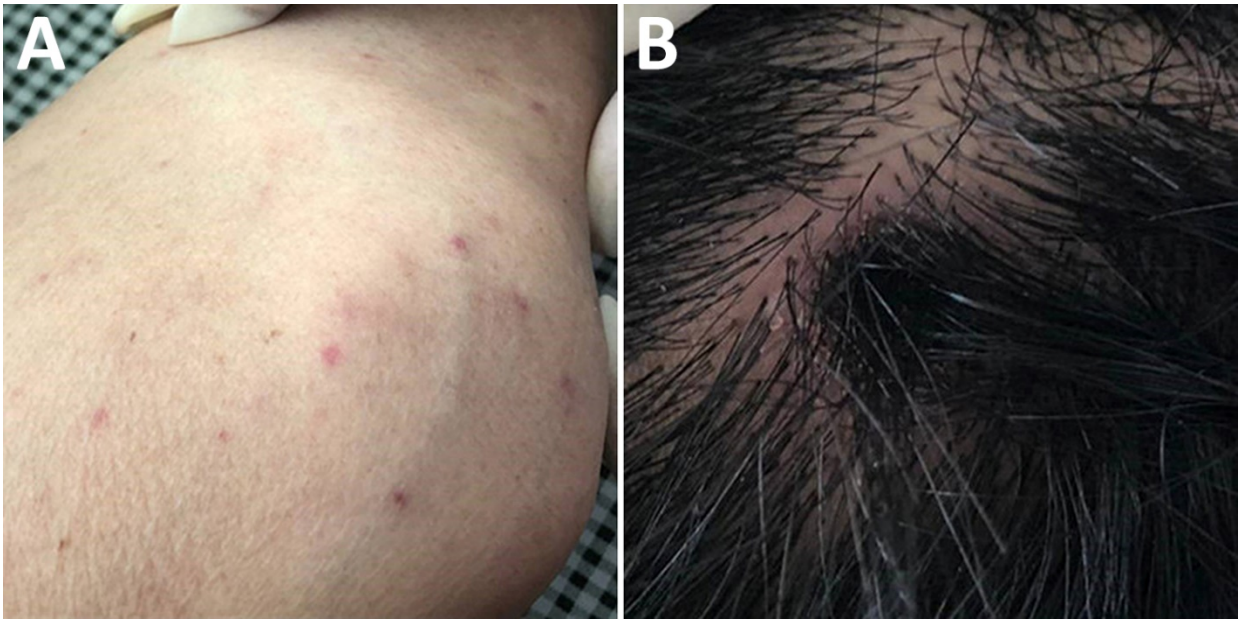
*BJ-90, *R. sibirica* subsp. *sibirica* BJ-90; 246, R. sibirica 246; HA-91, *R. sibirica* subsp. *mongolitimonae* HA-91.

†*glt*A, citrate synthase; *omp*A, outer membrane protein A; *omp*B, outer membrane protein B; 17kDa, 17kDa lipoprotein; *sca*1, surface cell antigen 1; *sac*4, surface cell antigen 4.

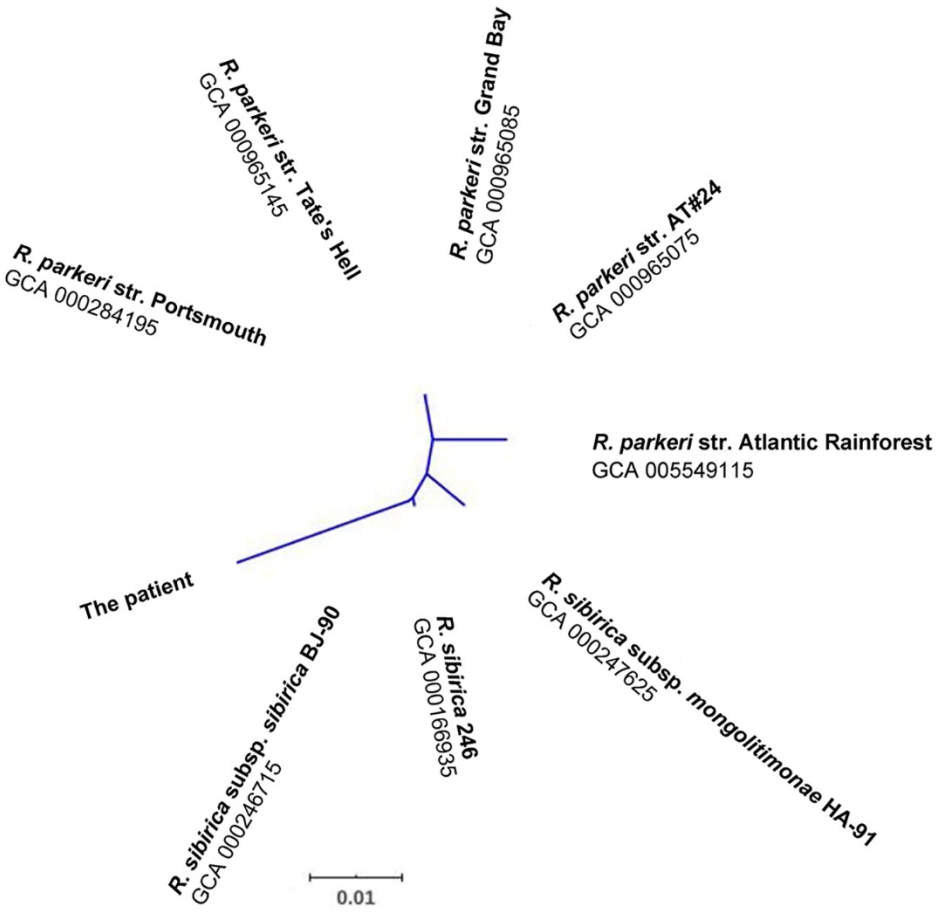
‡Reads of metagenomic sequencing.

§Total nucleotides of the rickettsial reads (266 reads, 100bp/read).

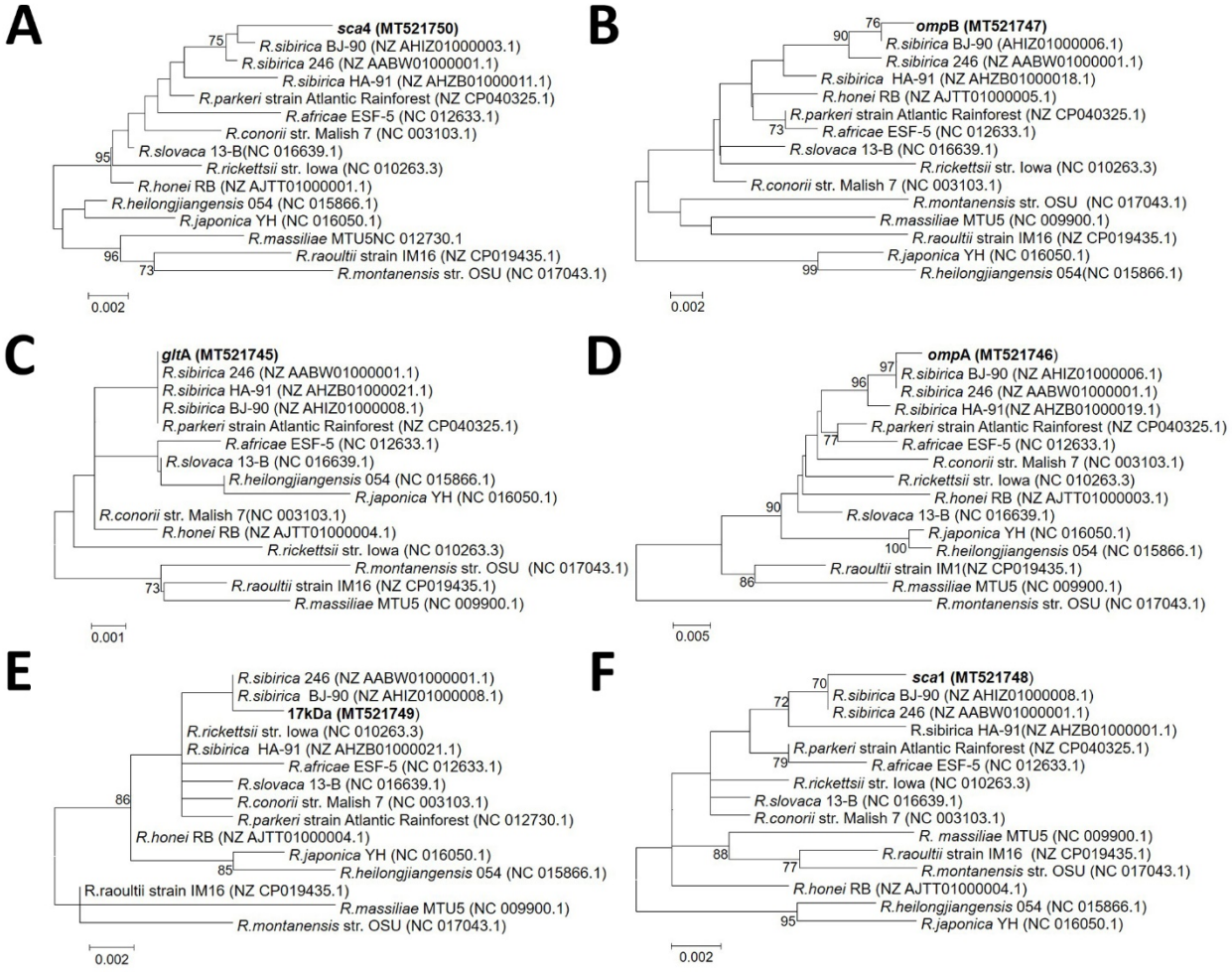
¶Accession number of the 21.6Gb metagenomic sequencing which were deposited in the National Genomic Data Center (NGDC).



Appendix Figure 1. Macular rash on the right knee area of the patient on 5 day (A) and the eschar at right posterior occipital region on day 16 (B) after recognition of a tick bite.



Appendix Figure 2. The causative agent of the Qinghai patient is closely related to *R.sibirica* subsp. *sibirica* BJ-90. An unrooted phylogenetic tree was generated on the basis of the concatenated rickettsial reads obtained from metagenomic sequencing of eschar DNA. Scale bar represents nucleotide distance.



Appendix Figure 3. Phylogenetic analysis of the causative agent. Phylogenetic tree was constructed on the basis of sequences of partial *glt*A, *omp*A, *omp*B,17kDa, *sca*1, and *sca*4. Amplicon sequences were aligned using MUSCLE within the MEGA 6 software. Phylogenetic relationships were analyzed using the neighbor-joining method with 1000 bootstrap replicates. Values >70 were indicated at the nodes. Bold represents the sequences obtained in this study. Scale bar indicates number of nucleotide substitutions.